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PROKARYOTIC IRON SUPEROXIDE DISMUTASE REPLACES CYTOSOLIC COPPER, ZINC SUPEROXIDE DISMUTASE IN PROTECTING YEAST CELLS AGAINST OXIDATIVE STRESS

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SUMMARY

The iron superoxide dismutase (FeSOD) gene of *Escherichia coli* was cloned in *Saccharomyces cerevisiae* cells deficient in copper,zinc superoxide dismutase (Cu,ZnSOD). FeSOD replaced Cu,ZnSOD in protecting the yeast cells against oxidative stress. In the recombinant strains the FeSOD gene, which was under the transcriptional control of the yeast phosphoglycerate kinase gene promoter, was functionally expressed at two different levels on episomal and centromeric plasmids. Despite suppression of methionine and lysine auxotrophy, the higher level of FeSOD activity was more beneficial to growth of the mutant yeast cells only when these were exposed to higher levels of oxidative stress induced by paraquat or 100% oxygen. In the presence of paraquat, there was a novel stimulation of FeSOD activity. This was associated with a marked increase in catalase activity, and a decrease in glutathione reductase activity.

INTRODUCTION

Antioxidant defense mechanisms have evolved within organisms to limit the levels of reactive oxygen species, such as the superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$), and to suppress oxygen toxicity (1). Among the defenses are enzymes such as superoxide dismutase (SOD[2]; EC 1.15.1.1) (2), catalase (CAT; EC 1.11.1.6) (3), cytochrome *c* peroxidase (CCP; EC 1.11.1.5) (4), glutathione peroxidase (GSH-Px; EC 1.11.1.9) (5), and glutathione reductase (GR; EC 1.6.4.2) (6).

The protective role of SOD against oxygen toxicity has been studied considerably in the eukaryote *Saccharomyces cerevisiae*. A yeast mutant lacking mitochondrial MnSOD was found to be hypersensitive to oxygen, and increasing concentrations of oxygen led to a progressive inhibition of growth (7). Cu,ZnSOD was also found to play an important role in protection against oxidative stress. *S. cerevisiae* strains lacking the cytosolic copper,zinc enzyme exhibited a number of oxygen-related growth defects (8-11).

Abbreviations: SOD, superoxide dismutase; CAT, catalase; CCP, cytochrome *c* peroxidase; GR, glutathione reductase; PGK, 3-phosphoglycerate kinase.

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In this communication, we show that *Escherichia coli* FeSOD expressed in its active form in the cytosol of *S. cerevisiae* cells deficient in Cu,ZnSOD protects the cells against physiological and induced oxidative stress. Our studies also show that the levels of activity of other antioxidant enzymes such as CAT, CCP and GR alter in accordance with the variation in level of the prokaryotic FeSOD in the yeast cells. Furthermore, we show that FeSOD activity is stimulated by paraquat under the experimental conditions.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions. The *E. coli* strain used in the cloning procedures was TG1 *(supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZΔM15])* obtained from Amersham International. The media used were (*i*) M9 minimal medium (glucose, 0.4%; $Na_2HPO_4 \cdot 7H_2O$, 1.28%; KH_2PO_4, 0.3%; NaCl, 0.05%; NH_4Cl, 0.1%) containing thiamine hydrochloride (5μg/ml); (*ii*) 2TY medium (Bacto-tryptone, 1.6%; Bacto yeast extract, 1%; NaCl, 0.5%); and (*iii*) LB medium (Bacto-tryptone, 1%; Bacto yeast extract, 0.5%; NaCl, 1%). Ampicillin was added at 50 μg/ml when required. For plates 1.5% agar was used and incubation was at 37 °C. Liquid cultures were maintained aerobically at 37 °C on a Controlled Environment Incubator Shaker (New Brunswick Scientific) at 200 rpm.

Yeast Strain and Culture Methods. The Cu,ZnSOD-deficient yeast strain *S. cerevisiae* Dscd2-4A *(α, leu1, his4, ura3, sod1-1)* (12) was supplied by T. Bilinski (Zamosc College of Agriculture, Zamosc, Poland). The culture media used were (*i*) YEPD (Bacto yeast extract, 1%; Bacto-peptone, 2%; glucose, 2%); (*ii*) minimal medium (Bacto yeast nitrogen base without amino acids, 0.67%; glucose, 2%; L-histidine, 100 μg/ml; adenine and L-methionine, 20 μg/ml; uracil, 25 μg/ml; L-lysine, 30 μg/ml; L-tryptophan, 40 μg/ml; L-leucine, 90 μg/ml, as required); and (*iii*) YPE medium (Bacto yeast extract, 1%; Bacto-peptone, 2%; ethanol, 3%). Aerobic growth in liquid cultures was maintained at 30 °C with constant shaking at 300 rpm.

Subcloning of FeSOD Gene in *E. coli* and Yeast. The *E. coli* FeSOD gene (13) flanked by the *S. cerevisiae* 3-phosphoglycerate kinase (*PGK*) gene promoter and transcription terminator was isolated by *Hin*d III digestion of the recombinant plasmid YEp/PGK-F (14). The 2.6 kilobase *Hin*d III fragment was ligated into the shuttle vector YCplac33 (15) (donated by G. Schatz, Basel University, Switzerland) at its *Hin*d III site to give rise to the recombinant plasmid YCplacSOD. The ligation experiments and sub-cloning of the FeSOD gene by means of the plasmid YCplacSOD in *E. coli* TG1 cells were according to Sambrook *et al.* (16). Transformation of *S. cerevisiae* Dscd2-4A cells by the shuttle vector YCplac33 (to give rise to the strain Dscd2-YC, used as control), and by the recombinant plasmids YCplacSOD (to give rise to the strain Dscd2-YC-F), and YEp/PGK-F (to give rise to the strain Dscd2-YEp-F), respectively, was carried out by the lithium acetate method according to Ito *et al.* (17). The methods used for preparing total cell extracts and membrane-free cytosolic extracts were as described previously (7, 18).

Measurement of Antioxidant Enzyme Activities. All enzyme assays were carried out on freshly prepared cell protein extracts. SOD activity was determined on total cell extracts according to McCord and Fridovich (2) and Ysebaert-Vanneste and Vanneste (19). FeSOD activity was measured on membrane-free cytosolic extracts from which mitochondria had been removed. CAT activity was determined according to the method of Aebi (20). CCP enzyme assays were carried out as specified by Yonetani (21). GR activity was assayed according to the procedure of Goldberg and Spooner (22), and for GSH-Px assays, the method of Flohè and Günzler (23) was used.

Response to Oxidative Stress. Cells from the *S. cerevisiae* strains Dscd2-YC, Dscd2-YC-F, and Dscd2-YEp-F cultured in both YEPD and minimal medium, were subjected to oxidative stress induced by 1 mM paraquat (Sigma). The cell growth was followed by measuring the optical density (OD) at 600 nm in a Perkin-Elmer Lambda 17 spectrophotometer after appropriate dilution of the cell cultures. Sensitivity of *S. cerevisiae* cells from the above strains to aerobic growth in

100% oxygen was also measured. Aliquots of diluted cell suspension (each containing ~100 cells) from each strain were plated on minimal medium. Open plates were sealed in a desiccator and flushed with 100% O_2 for 2 hr. The desiccator was then sealed and the plates were incubated for 4 days at 30 °C. Control plates which were not exposed to 100% oxygen, were also incubated for four days at 30 °C. Sensitivity to 100% oxygen was monitored by counting cell colonies.

Miscellaneous. DNA sequencing was carried out by the dideoxy method (24) using Sequenase I enzyme (United States Biochemical). Protein expression was studied by SDS/polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue (25). The UltroScan™XL laser densitometer and GelScan™XL software (Pharmacia LKB) were used for the quantitative evaluation of FeSOD bands on SDS/polyacrylamide gels. Published procedures were used to determine protein concentration (26) and activity of SOD in polyacrylamide gels (27).

RESULTS

Expression of Cloned *E. coli* FeSOD Gene in *S. cerevisiae* Cells Deficient in Cu,ZnSOD. The *E. coli* FeSOD gene was efficiently expressed in the two *S. cerevisiae* strains deficient in Cu,ZnSOD, Dscd2-YC-F and Dscd2-YEp-F. At early stationary phase in minimal medium, the level of FeSOD expression in Dscd2-YEp-F cells, where the FeSOD gene was cloned on the multicopy plasmid YEp/PGK-F, was found to be 7.5 times higher by gel scanning than in Dscd2-YC-F cells, where the gene was cloned on the centromeric plasmid YCplacSOD. This corresponds with results obtained from FeSOD assays where the enzyme activity in Dscd2-YEp-F cells was 7.9 times higher than in Dscd2-YC-F cells growing in minimal medium (Table 1A). The higher level of FeSOD activity in Dscd2-YEp-F cells when compared with Dscd2-YC-F was also observed on non-denaturing gels after activity staining (Fig. 1).

***E. coli* FeSOD Suppresses Methionine and Lysine Auxotrophies in Cu,ZnSOD-Deficient Yeast Cells.** *S. cerevisiae* cells carrying the *sod1-1* mutation which eliminates Cu,ZnSOD activity, require methionine and lysine for aerobic growth (8). Whereas cells from *S. cerevisiae* strains Dscd2-4A and Dscd2-YC (carrying only the centromeric plasmid YCplac33) exhibited methionine and lysine auxotrophy, cells from the recombinant strains Dscd2-YC-F and Dscd2-YEp-F, expressing cytosolic FeSOD instead of Cu,ZnSOD, did not require methionine and lysine for aerobic growth.

Cloned FeSOD Protects Cu,ZnSOD-Deficient Yeast Cells against Oxygen and Paraquat Toxicity. *S. cerevisiae* cells lacking Cu,ZnSOD are unable to grow in 100% O_2 in rich medium and are hypersensitive to superoxide-generating agents such as paraquat (8). In this study, the prokaryotic FeSOD replaced the eukaryotic Cu,ZnSOD in protecting the yeast cells cultured on minimal medium against oxidative stress induced by 100% oxygen. The percentage survival of cells after 2 hr exposure to 100% oxygen were 0% for Dscd2-YC, 18.3% for Dscd2-YC-F, and 78.3% for Dscd2-YEp-F.

Cloned FeSOD also protected the Cu,ZnSOD-deficient yeast cells against paraquat toxicity. In minimal medium, cells having cytosolic FeSOD grew faster than Dscd2-YC cells which carry only

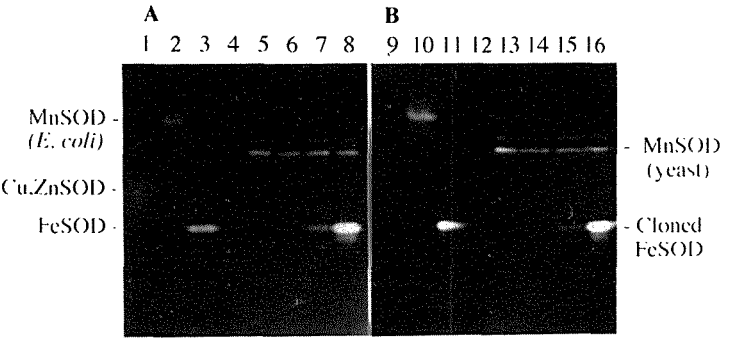

Fig. 1. Activity stain of *E. coli* FeSOD cloned on a centromeric and multicopy plasmid, respectively, in *S. cerevisiae* cells deficient in Cu,ZnSOD. Equal amounts (120 μg) of total cell protein from each of the *S. cerevisiae* strains Dscd2-4A (lanes 5 and 13), Dscd2-YC (lanes 6 and 14), Dscd2-YC-F (lanes 7 and 15), and Dscd2-YEp-F (lanes 8 and 16) were loaded in their respective lanes on two nondenaturing 10% (wt/vol) polyacrylamide gels (*A* and *B*). Lanes 1 and 9, 0.5 μg of bovine erythrocyte Cu,ZnSOD (Sigma); lanes 2 and 10, 5 μg of *E. coli* MnSOD (Sigma); lanes 3 and 11, 5 μg of *E. coli* FeSOD (Sigma). Gel in *B* was treated with 10 mM KCN to inactivate Cu,ZnSOD prior to the activity staining.

the centromeric plasmid without the cloned FeSOD gene, and Dscd2-YC-F cells which have less FeSOD activity than Dscd2-YEp-F cells, had the better growth rate (Fig. 2A(i)). Cell counts after growth to stationary phase confirmed the results.

In YEPD or minimal medium containing 1 mM paraquat, Dscd2-YEp-F cells containing the higher level of cytosolic FeSOD were better protected against oxidative stress induced by paraquat than Dscd2-YC-F cells. The *S. cerevisiae* Dscd2-YC cells deficient in cytosolic SOD were unable to grow in the presence of 1 mM paraquat (Fig. 2 B(ii) and A (ii)). Thus in the presence of 1 mM paraquat, as in the presence of 100% oxygen, the higher level of cytosolic SOD activity is more beneficial to cell growth.

In YPE medium, with ethanol as the non-fermentable carbon source, as with cells growing in minimal medium or YEPD medium in the absence of paraquat, Dscd2-YC-F cells with the lower level of cytosolic FeSOD grew better than Dscd2-YEp-F cells with the higher FeSOD expression and activity (Fig. 3). These results were also confirmed by cell counts. Dscd2-YC cells which have only MnSOD, were also able to grow after a long lag phase in YPE medium since the mitochondrial SOD offers protection against the $O_2^{\cdot}$ generated during aerobic respiration. However, the presence of cytosolic FeSOD in Dscd2-YC-F cells significantly enhanced the growth rate.

Stimulation of FeSOD activity by 1 mM Paraquat. The presence of 1 mM paraquat increased FeSOD activity from 3.1 to 17.2 U/mg of total cytosolic protein in Dscd2-YC-F cells, and from 24.4 to 41.9 U/mg of total cytosolic protein in Dscd2-YEp-F cells at late logarithmic to early stationary phase in minimal medium (Table 1A and 1B). When measurements were made at early to mid-logarithmic phase, there was a similar increase in FeSOD activity in the presence of 1

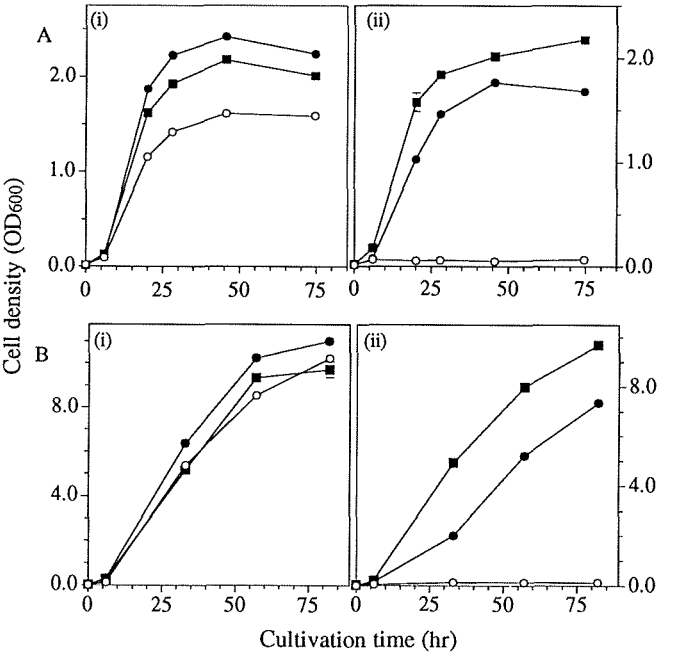

Fig. 2. Exponential growth curves of *S. cerevisiae* strains Dscd2-YC (Cu,ZnSOD-deficient mutant carrying only the centromeric plasmid YCplac33, ○), Dscd2-YC-F (Cu,ZnSOD-deficient mutant with *E. coli* FeSOD cloned on a centromeric plasmid, ●), and Dscd2-YEp-F (Cu,ZnSOD-deficient mutant with *E. coli* FeSOD cloned on a multicopy plasmid, ■). The cells were grown in liquid minimal medium (*A*) or YEPD medium (*B*), each type of medium containing 0 mM paraquat (i), or 1 mM paraquat (ii). Each point represents the mean of three independent determinations. Error bars are ± 1 S.D. and are shown where sufficiently large.

mM paraquat, from 6.3 to 27.1 U/mg of total cytosolic protein in Dscd2-YC-F cells, and from 78.5 to 125.6 U/mg of total cytosolic protein in Dscd2-YEp-F cells. In both recombinant strains FeSOD gene transcription was under the control of the *PGK* promoter.

Other Antioxidant Enzyme Response to Physiological and Induced Oxidative Stress. When growing in minimal medium containing 1 mM paraquat, the increase in FeSOD activity in Dscd2-YEp-F cells which had a better growth rate than Dscd2-YC-F cells (Fig. 2A(ii)), was accompanied by an increase in CAT and CCP activities and a decrease in GR activity (Table 1B). In YPE medium, a decrease in CAT, CCP and GR activities followed the increase in FeSOD activity in Dscd2-YEp-F cells (Table 1C). Contrary to when growing in minimal medium containing 1 mM paraquat, these cells had a slower growth rate in YPE medium than Dscd2-YC-F cells (Fig. 3) with a lower level of FeSOD activity but markedly higher levels of CAT and CCP activities.). No GSH-Px activity could be detected in the recombinant strains, in accordance with the results of Smith and Shrift (28) who reported the absence of GSH-Px activity in yeast.

DISCUSSION

In this study, *E. coli* FeSOD has been found to replace Cu,ZnSOD in protecting yeast cells against oxidative stress. The sub-cloning of the FeSOD gene either on a multicopy or centromeric plasmid in *S. cerevisiae* Dscd2-4A cells deficient in Cu,ZnSOD, produced two recombinant strains with very different levels of FeSOD enzyme activity. The higher level of FeSOD activity conferred on Dscd2-YEp-F cells a better resistance to paraquat and 100% oxygen, whereas in the absence of paraquat or when growth was in YPE medium, Dscd2-YC-F cells with the lower FeSOD activity had the better growth rate. This seems to indicate that a considerable increase in FeSOD activity is beneficial to the mutant yeast cells only when exposed to induced higher levels of oxidative stress. This finding appears to extend to yeast the current hypothesis, summarized by McCord (29), that in a normal healthy cell, an optimal balance exists between superoxide production and superoxide scavenging, and that overscavenging of the radical may possibly lead to cellular damage.

In the presence of 1 mM paraquat, there was a significant increase in FeSOD activity in both Dscd2-YC-F and Dscd2-YEp-F cells (Table 1A and 1B). In the two strains the FeSOD gene was under the transcriptional control of the *PGK* promoter. Further work is in progress to investigate this stimulatory effect of paraquat. This may lead to a better understanding of the response of yeast cells to oxidative stress.

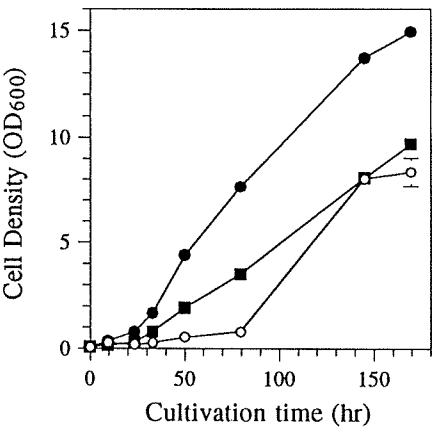

Fig. 3. *S. cerevisiae* Dscd2-YC-F cells with *E. coli* FeSOD cloned on a centromeric plasmid, have a growth advantage over Dscd2-YEp-F cells with FeSOD cloned on a multicopy plasmid, when grown on a nonfermentable carbon source. Cells from the *S. cerevisiae* strains Dscd2-YC (Cu,ZnSOD-deficient mutant carrying only the centromeric plasmid YCplac33, ○), Dscd2-YC-F (Cu,ZnSOD-deficient mutant with *E. coli* FeSOD cloned on a centromeric plasmid, ●), and Dscd2-YEp-F (Cu,ZnSOD-deficient mutant with *E. coli* FeSOD cloned on a multicopy plasmid, ■) were grown in YPE medium at 28 °C. Each point represents the mean of three independent determinations. Error bars are ± 1 S.D. and are shown where sufficiently large.

Table 1. Antioxidant enzyme activities of recombinant *S. cerevisiae* strains

Cells were grown in (*A*) minimal medium, (*B*) minimal medium containing 1 mM paraquat, (*C*) yeast extract, peptone and ethanol (YPE). Minimal medium contained glucose as the carbon source.

A

Yeast Strains	Total SOD	FeSOD	CAT	CCP	GR
Dscd2-YC	6.0 ± 0.4		18.6 ± 0.3	0.68 ± 0.08	0.22 ± 0.007
Dscd2-YC-F	10.6 ± 0.3	3.1 ± 0.1	17.7 ± 0.8	0.71 ± 0.08	0.17 ± 0.002
Dscd2-YEp-F	54.6 ± 2.3	24.4 ± 0.9	19.6 ± 1.7	0.77 ± 0.04	0.18 ± 0.002

B

Yeast Strains	Total SOD	FeSOD	CAT	CCP	GR
Dscd2-YC-F	28.5 ± 0.4	17.2 ± 0.6	34.1 ± 1.1	0.31 ± 0.0	0.20 ± 0.002
Dscd2-YEp-F	66.8 ± 2.1	41.9 ± 1.2	54.3 ± 9.8	0.69 ± 0.02	0.11 ± 0.002

C

Yeast Strains	Total SOD	FeSOD	CAT	CCP	GR
Dscd2-YC	25.6 ± 1.5		53.7 ± 3.6	1.9 ± 0.10	0.25 ± 0.003
Dscd2-YC-F	31.1 ± 1.3	4.1 ± 0.0	50.4 ± 5.0	1.43 ± 0.28	0.16 ± 0.006
Dscd2-YEp-F	89.4 ± 3.7	53.7 ± 3.0	19.3 ± 3.0	0.94 ± 0.03	0.11 ± 0.001

Cells were harvested between late logarithmic and early stationary phase. There was no growth of Dscd2-YC cells on minimal medium containing 1 mM paraquat. Specific activity was determined in triplicate on at least three independent extracts. Cytochrome *c* peroxidase (CCP) activity was normalized to 20 μM cytochrome *c* concentration in the assay. Data are given with S.E. Specific activities of total cellular superoxide dismutase (SOD), catalase (CAT), CCP and glutathione reductase (GR) are quoted as U/mg total cell protein while iron superoxide dismutase (FeSOD) activity is quoted as U/mg membrane-free cytosolic protein.

Modifications in other antioxidant enzyme activities corresponding to variations in FeSOD activity levels, were also found in this work. In YPE medium, the considerable increase in FeSOD activity in Dscd2-YEp-F cells was accompanied by a decrease in CAT, CCP and GR activities (Table 1C). This could be due to some unbalance in the protein machinery of the yeast cells because of excessive production of FeSOD, as was observed by Liochev and Fridovich (30) in *E. coli*. Also in YPE medium, Dscd2-YC-F cells (with the lower level of FeSOD activity, and higher activity levels of CAT and CCP) had a significantly better exponential growth rate than Dscd2-YEp-F cells with the higher level of FeSOD activity (Fig. 3). Hence a lower level of cloned cytosolic FeSOD activity, together with higher activity levels of CAT and CCP, seem to confer a growth rate advantage on Dscd2-YC-F cells by counteracting overproduction of H_2O_2 and thus minimizing the sensitization of the cells to oxidative stress.

In minimal medium containing 1 mM paraquat, there was a marked increase in CAT activity, and a decrease in GR activity corresponding with the increase in FeSOD activity in Dscd2-YEp-F cells (Table 1B). The activity increases in FeSOD and CAT protect the yeast cells against the induced production of the superoxide radical, whilst controlling the H_2O_2 level within the cells.

Also a reduction in GR activity means that there is less depletion of the NADPH reserves of the cells caused by reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) at the expense of NADPH (31). These combined antioxidant enzyme levels conferred a growth advantage to Dscd2-YEp-F cells over Dscd2-YC-F cells which have lower activity levels of FeSOD, CAT and CCP and a higher GR activity (Table 1B). Ensuring that levels of ATP and NADPH are maintained subsequent to induced oxidative stress seems to be an important antioxidant response. These results support one of the proposals by Winterbourn (32) that superoxide can cause oxidative stress to the cell by depleting cellular energy reserves by consumption of NADPH in free radical chain reactions involving GSH.

In the presence of 1 mM paraquat, CCP activity levels are either reduced as in Dscd2-YC-F cells, or do not undergo any appreciable change as in Dcsd2-YEp-F cells when comparison is made with growth of the same cells on minimal medium without paraquat (Table 1A and 1B). Therefore, it is mainly CAT which controls the H_2O_2 level within the cells. This suggests that $O_2^{\cdot}$ and hence H_2O_2 may be generated by paraquat also within the cytosol of the yeast cells. Nonspecific reductases that can act on paraquat have been identified in *S. cerevisiae* (33).

Observations from this study have shown that prokaryotic FeSOD efficiently replaces eukaryotic Cu,ZnSOD in the cytosol of yeast cells. Despite suppression of methionine and lysine auxotrophy, the increased activity levels of the cloned FeSOD are beneficial to cellular growth only during exposure to induced higher levels of oxidative stress, thus supporting the hypothesis of an optimal balance between superoxide production and superoxide scavenging under normal conditions.

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REFERENCES

1. Halliwell, B., and Gutteridge, J.M.C. (1988) ISI Atlas Sci. Biochem. 1, 48-52.
2. McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
3. Chance, B., Sies, H., and Boveris, A. (1979) Physiol. Rev. 59, 527-605.
4. Yonetani, T., and Ray, G.S. (1966) J. Biol. Chem. 241, 700-706.
5. Cohen, G., and Hochstein, P. (1963) Biochemistry 2, 1420-1428.
6. Schirmer, R.H., Krauth-Siegel, R.L., and Schulz, G.E. (1989) in Glutathione: Chemical, Biochemical and Medical Aspects, Part A, (Dolphin, D., Poulson, R., and Avramovic, O., Eds.) pp. 553-596, Wiley, UK.
7. van Loon, A.P.G.M., Pesold-Hurt, B., and Schatz, G. (1986) Proc. Natl. Acad. Sci. USA 83, 3820-3824.
8. Bilinski, T., Krawiec, Z., Liczmanski, A., and Litwinska, J. (1985) Biochem. Biophys. Res. Commun. 130, 533-539.
9. Chang, E.C., Crawford, B.F., Hong, Z., Bilinski, T., and Kosman, D.J. (1991) J. Biol. Chem. 266, 4417-4424.
10. Gralla, E.B., and Valentine, J.S. (1991) J. Bacteriol. 173, 5918-5920.

11. Liu, X.F., Elashvili, I., Gralla, E.B., Valentine, J.S., Lapinskas, P., and Culotta, C. (1992) J. Biol. Chem. 267, 18298-18302.
12. Bilinski, T., Litwinska, J., Krawiec, Z., and Achremowicz, B. (1993) Acta Microbiol. Pol. 42, 101-104.
13. Carlioz, A., Ludwig, M.L., Stallings, W.C., Fee, J.A., Steinman, H.M., and Touati, D. (1988) J. Biol. Chem. 263, 1555-1562.
14. Balzan, R., Bannister, W.H., Hunter, G.J., and Bannister, J.V. (1995) Proc. Natl. Acad. Sci. USA 92, 4219-4223.
15. Gietz, D.R., and Sugino, A. (1988) Gene 74, 527-534.
16. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
17. Ito, H., Jukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163-168.
18. Daum, G., Böhni, P.C., and Schatz, G. (1982) J. Biol. Chem. 257, 13028-13033.
19. Ysebaert-Vanneste, M., and Vanneste, W.H. (1980) Anal. Biochem. 107, 86-95.
20. Aebi, H.E. (1983) in Methods of Enzymatic Analysis, 3rd Ed., Vol. 3, (Bergmeyer, H.U., Ed.) pp. 273-286, Verlag Chemie, Deerfield Beach, FL.
21. Yonetani, T. (1967) in Methods in Enzymology, Vol. X, (Estabrook, R.W., and Pullman, M.E., Eds) pp. 336-339, Academic Press, NY.
22. Goldberg, D.M., and Spooner, R.J. (1983) in Methods of Enzymatic Analysis, 3rd Ed., Vol. 3, (Bergmeyer, H.U., Ed.) pp. 258-265, Verlag Chemie, Deerfield Beach, FL.
23. Flohè, L., and Günzler, W.A. (1984) in Methods in Enzymology, Vol. 105, (Packer, L., Ed.) pp. 114-121, Academic Press, NY.
24. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
25. Sedmak, J.J., and Grossberg, S.E. (1977) Anal. Biochem. 79, 544-552.
26. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
27. Beauchamp, C., and Fridovich, I. (1971) Anal. Biochem. 44, 276-287.
28. Smith, J., and Shrift, A. (1978) Comp. Biochem. Physiol. 63B, 39-44.
29. McCord, J.M. (1995) Proc. Soc. Exp. Biol. Med. 209, 112-117.
30. Liochev, S.I., and Fridovich, I. (1992) Arch. Biochem. Biophys. 294, 138-143.
31. Sinet, P.M., Michelson, A.M., Bazin, A., Lejeune, J., and Jerome, H. (1975) Biochem. Biophys. Res. Commun. 67, 910-915.
32. Winterbourn, C.C. (1993) Free Rad. Biol. Med. 14, 85-90.
33. Gralla, E., and Kosman, D.J. (1992) Adv. Genet. 30, 251-319.